RESEARCH ARTICLE

MAGED4 Expression in Glioma and Upregulation in Glioma Cell Lines with 5-Aza-2'-Deoxycytidine Treatment

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Abstract

Melanoma-associated antigen (MAGE) family genes have been considered as potentially promising targets for anticancer immunotherapy. MAGED4 was originally identified as a glioma-specific antigen. Current knowledge about MAGED4 expression in glioma is only based on mRNA analysis and MAGED4 protein expression has not been elucidated. In the present study, we investigated this point and found that MAGED4 mRNA and protein were absent or very lowly expressed in various normal tissues and glioma cell line SHG44, but overexpressed in glioma cell lines A172,U251,U87-MG as well as glioma tissues, with significant heterogeneity. Furthermore, MAGED4 protein expression was positively correlated with the glioma type and grade. We also found that the expression of MAGED4 inversely correlated with the overall methylation status of the MAGED4 promoter CpG island. Furthermore, when SHG44 and A172 with higher methylation were treated with the DNA demethylating agent 5-aza-2'-deoxycytidine (5-AZA-CdR) reactivation of MAGED4 mRNA was mediated by significant demethylation in SHG44 instead of A172. However, 5-AZA-CdR treatment had no effect on MAGED4 protein in both SHG44 and A172 cells. In conclusion, MAGED4 is frequently and highly expressed in glioma and is partly regulated by DNA methylation. The results suggest that MAGED4 might be a promising target for glioma immunotherapy combined with 5-AZA-CdR to enhance its expression and eliminate intratumor heterogeneity.

Keywords: MAGED4 - expression - methylation - glioma

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Introduction

Gliomas are the most common malignant tumors in the central nervous system, accounting for approximately 50% of primary brain tumors (Zeybek et al., 2013). These tumors are characterized by rapid invasive growth into surrounding brain parenchyma, and thus result in a poor prognosis, despite advances in surgical resection, radiotherapy and chemotherapy (Ohgaki et al., 2005; Wu et al., 2012). Clearly, novel and innovative therapeutic strategies are required to improve current treatment strategies.

Immunotherapy is an attractive approach among novel therapeutic strategies (Chi et al., 1997; Prins et al., 2003; Ahmed et al., 2014). Although brain has long been considered an immunologically privileged site, many recent studies have demonstrated that immune responses frequently occur in this compartment with or without the blood brain barrier breakdown (Sampson et al., 1996; Becher et al., 2000). Therefore, immunotherapeutic approaches are feasible for glioma patients. Recently, some immunotherapeutic approaches have been explored as complementary treatments for patients with glioma, but satisfactory antitumor effects were not observed (Yang et al., 2003). This might be partially attributed to the lack of a well-defined glioma-specific antigen.

MAGE is a large gene family, in which some genes encode tumor-associated antigens with characteristics of broad expression in various tumors but restricted in normal tissues, and recognizing by cytotoxic T lymphocytes (CTLs) (Inoue et al., 1995; Wischnewski et al., 2006). Some of MAGE antigens and their epitope peptides constitute important targets for antitumor immunotherapy with a number of clinical studies already completed or underway (Duffour et al., 1999; Rosenberg et al., 2004; Morse et al., 2005). MAGED4 is one of member in MAGE family. It was originally termed as MAGEE1 and identified as a glioma-specific antigen by serial analysis of gene expression (SAGE) (Sasaki et al., 2001). MAGED4 mRNA is predominantly expressed in glioma, but not

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expressed in normal tissues except brain and ovary (Sasaki et al., 2001). MAGED4 mRNA was also reportedly up-regulated 2.6 to 15 folds in glioblastoma, relatively to cultured human astrocytes (Sasaki et al., 2001). Then, another study (Kramer et al., 2005) got the similar result for MAGED4 mRNA expression. A MHC class I ligand from MAGED4 presented by HLA-A on tumor tissue was also identified in this report (Kramer et al., 2005). Recent studies have shown that MAGED4 contributes to proliferation, migration, and invasion of tumor cells in breast cancer and oral squamous cell carcinoma (Germano et al., 2011; Chong et al., 2012). To some extent, these preliminary findings have been suggested that MAGED4 may play an important role in the progression of tumors and may be a potentially promising target for tumor treatment.

However, researches about MAGED4 gene expression in glioma were only based on mRNA level. MAGED4 protein expression has not been elucidated including its expression mechanism. Although a presumptive CpG island of MAGED4 around the transcriptional site has been reported (Kawano et al., 2001), its methylation status is not yet known so far. Generally, most MAGE family genes present CpG island in the promoter region, and demethylation of this site is related to induce expression of some genes in this family (Weber et al., 1994; Serrano et al., 1996; Wischnewski et al., 2006). This implies that MAGED4 could be an DNA methylation-mediated regulation gene. In the present study, we first examined various normal tissues and gliomas for the expression pattern of MAGED4 in both mRNA and protein. Furthmore, possible relationship between MAGED4 protein expression and clinicopathological parameters of glioma patients was investigated. We then sought to determine if treatment of glioma cell lines with 5-AZA-CdR, a demethylating agent, would induce or upregulate MAGED4 expression.

Materials and Methods

Cell lines and tissue samples

Human glioma cell lines SHG44, A172, U251 and U87-MG were obtained from Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine at 37°C in a 5% CO_2 humidified atmosphere.

Primary glioma tissues were available from the First Affiliated Hospital of Guangxi Medical University, approved by Hospital Ethic Review Committee with written informed consent from patients. Normal brain specimens were obtained in patients undergoing resection of nonneoplastic brain lesions as part of the treatment of their underlying condition (trauma). RT-PCR and qRT-PCR analysis were used for detecting 7 gliomas with 4 low-grade astrocytomas (WHO grade I-II) and 3 high-grade astrocytomas/glioblastomas (WHO grade IV) (Louis et al., 2007) as well as a panel of normal tissues, which including 2 normal brain and 1 case of breast, ureter, kidney, fallopian tube, testis, fat, thyroid, uterus, appendix, stomach, skin, prostate, cervix, ileum, tonsil and colon. These normal tissues were available from the Tissue Bank of the Department of Histology and Embryology at Guangxi Medical University.

71 paraffin-embedded giloma tissue sections (31 low-grade astrocytomas and 40 high-grade astrocytomas/glioblastomas; patient age range, 3-68 years; average patient age, 41.1 years; 40 men and 31 women) were processed for immunohistochemistry analysis. Normal tissue microarray chip was purchased from Alenabio (Xi'an, China). The chip included 5 cases of brain, breast, kidney, testis, stomach, skin, prostate, cervix, colon, rectum, small intestine, ovary, lymph node, thymus, bone marrow, esophagus, liver, pancreas and spleen.

Demethylation treatment

Cells were counted and seeded at a density of 0.5×10^5 cells/ml in a T75 tissue culture flask and grown overnight. On the day of treatment, the medium was removed and replaced with fresh medium containing 1μM 5-AZA-CdR (Sigma, USA), every 24 hours for 5 days. At the end of treatment, the cells were cultured in fresh media without 5-AZA-CdR for an additional 48 hours. Control was set up with the same volume of culture medium without 5-AZA-CdR.The cells were harvested and prepared for DNA, RNA and protein analysis.

RT-PCR and qRT-PCR analysis

Total RNA was extracted from all tissues and cell lines and was reverse transcribed into cDNA. cDNAs were then tested for integrity by amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. RT-PCR was carried out with MAGED4 specific primers (forward primer: 5'- CAGGATGGGAGGCAAGAGGACC-3' and reverse primer: 5'-CCAAGGAGGCGAGCTGAGGAGT-3'). The cycling parameters were as following: initial denaturantion at 94°C for 5 min followed by 30 sec at 94°C, 30 sec at (64°C for MAGED4, 55°C for GAPDH), and 30 sec at 72°C for 35 cycles, and a final extension for 10 min at 72°C. The expression of MAGED4 was counted as positive, only if the RT-PCR reaction repeated at least twice with same result.

qRT-PCR was performed on the StepOneTM Real-Time PCR System (Applied Biosystems, USA) using SYBR Green I (Roche, Germany). MAGED4 primers for qRT-PCR was reported previously (Sasaki et al., 2001). The following cycling parameters were used: initial denaturation at 95°C for 10 min, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. Dissociation curves were performed to confirm specific product amplification. Each cDNA sample was run as triplicates. MAGED4 mRNA expression was normalized to GAPDH and fold differences were determined using the $2^{-\Delta\Delta CT}$ method.

Immunohistochemistry and immunocytochemistry

Immunohistochemistry (IHC) was performed with minor modification as previously report by luo et al (Luo et al., 2013). In brief, tissue sections were deparaffinized and heated in ethylene diamine tetraacetic acid (EDTA, pH8.0) for antigen retrieval. After the inactivation of endogenous peroxidase, the sections were

treated with normal goat serum for blocking and then immunostained with anti-MAGED4 polyclonal antibody (1:500 dilution, Santa Cruz Biotechnology, USA)) overnight at 4°C. Negative controls using rabbit serum collected before immunization were also incubated in parallel. Subsequently, horseradish peroxidase-conjugated goat anti-rabbit IgG (ZSGB-BIO, China) was added as the secondary antibody. Immunoreactivity was visualized with 3, 3'-diaminobenzidine (DAB) (Maixin Biotechnology, China) followed by haematoxylin counterstain. For immunocytochemistry (ICC), cells were seeded in the wells of 24-well plate containing gelatin-coated coverslip. After 24 hours, the cells were fixed with methanol for 10 mininutes and permeabilized with PBS containing 0.3% Triton X-100 for 10 minutes at room temperature. The following steps from inactivation of endogenous peroxidase to counterstaining were performed as described for immunohistochemistry.

Positive immunoreactivity was assessed by two independent pathologists who did not know patients' clinical information and recorded semi-quantitatively according to the staining intensity and the percentage of positive cells. The staining intensity was quantified using the following scores: 0=negative, 1=weak staining, 2= moderate staining, 3=strong staining. The percentage of positive tumor cells was defined using the following scores: 0=0-5%, 1=6-25%, 2=26-50%, 3=51-75%, 4=76-100%. The final immunoreactive score was determined by the sum of both points: 0-1: negative expression (-), 2-3: weak expression (+), 4-5: moderate expression (++), 6-7: strong expression (+++) (Thaker et al., 2004; Ito et al., 2006).

Western blot analysis

Western blotting was performed with minor modification as previously described (Cen et al., 2012; Zhang et al., 2013). In brief, total protein was extracted from untreated and 5-AZA-CdR-treated glioma cells using a Total Protein Extraction Kit (KeyGen, China) according to the manufacturer's instructions. Then the protein was separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, USA). After blocking with 5% non-fat dry milk, the membrane was sequentially incubated with the primary antibody MAGED4 (Santa Cruz Biotechnology, USA) at a 1:500 dilution overnight at 4°C. A IRDye® 800CW-labelled goat anti-rabbit antibody (LI-COR Biosciences, Germany) was used as second antibody. The protein expression was represented by the band intensity analyzed by LI-COR Odyssey infrared imaging system (LI-COR Biosciences, Germany). the housekeeping gene GAPDH served as an internal control for normalization.

Bisulfate conversion and DNA sequencing analysis

Genomic DNA was isolated from the indicated cell lines and normal brain tissues using TIANamp Blood DNA Kit (Tiagen, China). The quantity and quality of DNA was measured using a NanoDrop 2000 Spectrophotometer (Thermo Scientific). 1μg of DNA was treated with sodium bisulfite using an CpGenome™ DNA Modification Kit (Chemico, USA) following the manufacturer's instructions. Subsequently, modified DNA was subjected to PCR to amplify the MAGED4 promoter CpG island identified by EMBOSS CpGPlot (http://www.ebi.ac.uk/Tools/emboss/cpgplot/index.html) and MethPrimer (http://www.urogene.org/methprimer/index1.html).

Two primer pairs were designed to amplify two part of the CpG island, which named as region 1 and region 2, using Methyl Primer Express Software version 1.0 and the online MethPrimer software (http://www.urogene.org/methprimer/index1.html) respectively. The primer sequences were as follows: Primer 1: 5'-GTTGTATTGTAGGGTATGAGTGT-3' (forward) and 5'-CCTACTACTACCAACACAACAACC-3' (reverse); Primer 2: 5'-GGGTTGTTGTGTTGGTAGTAGTAG-3' (forward) and 5'-TCTAAACCTTAAATCTAATAAAAT TCC-3' (reverse). The EpiTect PCR Control DNA Set (Qiagen, Germany) was served as negative and positive controls. PCR was performed with hotStar Taq DNA polymerase (Takara, China) using the following cycling conditions: (a) initial denaturation (15 min at 95°C); (b) 5 cycles of denaturation (30 sec at 94°C), annealing (Primer 1, 30 sec at 65°C; Primer 2, 30 sec at 60°C) and elongation (90 sec at 72°C); (c) 5 cycles of denaturation (30 sec at 94°C), annealing (Primer 1, 30 sec at 60°C; Primer 2, 30 sec at 55°C) and elongation (90 sec at 72°C); (d) 35 cycles of denaturation (30 sec at 94°C), annealing (Primer 1, 30 sec at 55°C ; Primer 2, 30 sec at 50°C) and elongation (90 sec at 72°C); (e) final extension (10 min at 72°C).

PCR products were gel purified using TIANgel Midi Purification Kit (Tiagen, China), and were ligated into the pGEM-T Easy Vector (Promega, USA). DH5a chemically competent cells (BioTeck Corporation, China) were transformed with the ligation products.Ten positive clones for each sample were randomly selected and sequenced at SinoGenoMax Co. Ltd (Beijing, China).

Statistical analysis

Statistical analyses were conducted using SPSS version 16 (SPSS, USA). The association between MAGED4 protein and clinicopathological parameters was determined by χ^2 test or fisher's exact test. Differences of MAGED4 expression were analyzed using One-Way ANOVA and Student-Newman-Keuls test. Differences of promoter methylation was evaluated by χ^2 test. Statistical significance was defined as $p<0.05$.

Results

MAGED4 expression in normal adult tissues and glioma

We first analyzed normal tissues and glioma for MAGED4 expression. As shown in Figure 1A, MAGED4 mRNA expression was positive in 11 out of 19 normal tissues, in all of glioma tissues tested and 3 out of 4 glioma cell lines with conventional RT-PCR. A heterogenous expression pattern was demonstrated in those tissues and cell lines tested. Therefore, the samples with MAGED4 mRNA were selected for further qRT-PCR analysis to reveal the quantity of MAGED4 mRNA. Through qRT-PCR, MAGED4 mRNA was found to be very low in

Figure 1A

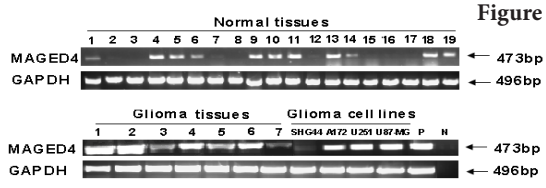

Figure 1B

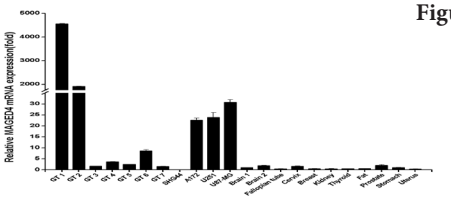

Figure 1. The Expression of MAGED4 in Normal Tissues and Glioma. A) Conventional RT–PCR analysis of MAGED4 mRNA. GAPDH was used as an internal control for the parallel PCR analysis of the same sample. cDNA from a glioma tissue with high-MAGED4 expression and PBS were served as positive controls (P) and negative controls (N), respectively. Lanes in a panel of normal tissues indicated as following: 1, Fallopian tube; 2, Tonsil; 3, Skin; 4, Cervix; 5, Breast; 6, Kidney; 7, Testis; 8, Muscle; 9, Thyroid; 10, Fat; 11, Prostate; 12, Ureter; 13, Stomach; 14, Uterus; 15, Colon; 16, Appendix; 17, Ileum; 18 and 19, Brain from different indivdual. Lanes in a panel of glioma tissues were as following: 2, 3, 6, 7: Low-grade astrocytomas; 1, 4, 5: High-grade astrocytomas/ Glioblastomas. Arrow indicates the RT-PCR products with base pairs (bp). **B)** qRT-PCR analysis of MAGED4 mRNA. MAGED4 expression was normalized to GAPDH. All data shown are the mean±SE of three independent experiments. GT: glioma tissues

normal tissues, whereas most of glioma tissues highly expressed MAGED4 mRNA. The level of MAGED4 mRNA in glioma tissues varied from 1.5- to 4560-fold relative to normal brain. In glioma cell lines tested, MAGED4 mRNA was almost absent in SHG44, whereas highly expressed in others, among which U87-MG exhibited the strongest expression (Figure 1B).

To clarify the expression of MAGED4 protein in normal tissues and glioma, IHC and ICC were analyzed. The result showed that all of normal tissues failed to detect MAGED4 protein (Figure 2A, 2B, 2C and 2D), but 66 out of 71 (93%) glioma tissues exhibited MAGED4 protein expression with different staining intensity (Figure 2F, 2G and 2H). Among these glioma tissue sections, negative (Figure 2E), weak (Figure 2F), moderate (Figure 2G) and strong (Figure 2H) were showed in five, sixteen, twenty-nine and twenty-one samples, respectively. Notably, although glioma tissues tested demostrated a high frequency of MAGED4 protein, heterogeneous expression of MAGED4 protein was still present in a significant number of glioma samples and resulted in individual positive cells or foci of stained cells (Figure 2F and 2G). In glioma cell lines MAGED4 protein expression was consistent with their mRNA expression. The distribution of MAGED4 protein was mostly in the cytoplasm and nucleus of tumor cells.

Associations between the MAGED4 expression and clinicopatholocial parameters in glioma

The association between MAGED4 protein expressions and the clinicopathological features were statistically

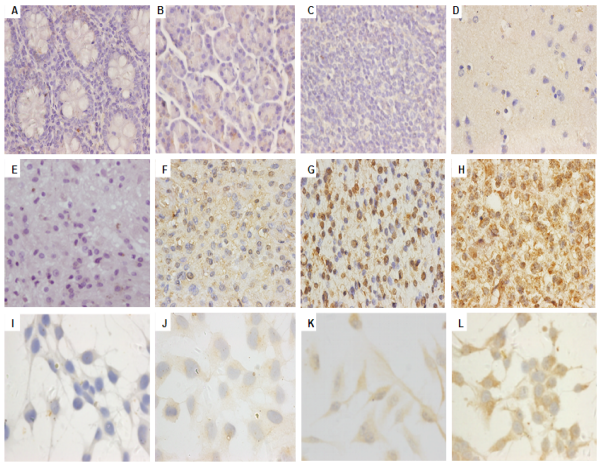

Figure 2. Immunohistochemical and Immunocytochemical Staining of MAGED4 Protein. No positive reactivity was observed in normal tissue (A: Colon, B: Pancreas, C: Lymph node and D: Brain). Immunoreactivity of MAGED4 protein was shown in glioma tissues E (Low-grade astrocytoma, Negative), F (High-grade astrocytom/ Glioblastoma, weak), G (Low-grade astrocytoma, moderate) and H (High-grade astrocytom/Glioblastoma, strong), respectively. Different immunoreactivity of MAGED4 protein were also shown in glioma cell lines I (SHG44), J (A172), K (U251) and L (U87-MG). Original magnification, ×400

Table 1. Correlation of MAGED4 Protein Expression with Clinicopathological Parameters in Glioma

Clinicopathological parameters		Positive/Total (%)	*p* value
Gender	Male	36/40(90.0)	0.378
	Female	30/31(96.8)	
Age(year)	≤41	30/32(93.8)	1
	>41	36/39(92.3)	
Histological type and grade			
Low-grade astrocytom (WHO I- II)		26/31(83.9)	0.013
High-grade astrocytom/ Glioblastoma (WHO IV)		40/40(100.0)	

evaluated. As shown in Table 1, MAGED4 protein expression was positively associated with the glioma type and grade. MAGED4 protein expression was more frequent in high-grade glioma (gliomblastoma) compared to the low-grade astrocytoma. No correlation was found between MAGED4 protein expression and any other clinicopathological features of glioma patients.

MAGED4 methylation status in glioma cell lines

We determined the methylation status of CpG sites in MAGED4 promoter region by bisulfite sequencing analysis in glioma cell lines. Two normal brain tissues with MAGED4-absent/low mRNA expression were served as normal controls. The methylation status of 37 CpG sites of MAGED4 promoter from 10 individual clones is shown in Figure 3. The control DNA was also tested in paralle, which demonstrated 97% methylation in methylation-positive control (Me) and 2% methylation in unmethylation-positive control (Unme), respectively, indicating that bisulfite sequencing analysis was properly performed. The percentage of overall methylation in glioma cells (from 36% to 5%) was lower than that in two

normal brain tissues (57.3% and 52.4%, respectively). The SHG44 with negative MAGED4 expression showed the highest percentage of overall methylation (36%). Wherease, U87-MG with the strongest MAGED4 expression exhibited the lowest percentage of overall methylation (5%). In addition, CpG sites within region 2 in SHG44 and A172 were highly methylated compared to other cells. Through this analysis an inverse correlation between the methylation of CpG sites and mRNA expression of MAGED4 was established in glioma cell lines tested.

Influence of 5-AZA-CdR on expression and methylation status of MAGED4

We assessed whether MAGED4 could be induced by 5-AZA-CdR treatment in SHG44 and A172 cells 5-AZA-CdR treatment in 2 glioma cell lines (SHG44 and A172) with higher methylation status of MAGED4. The qRT-PCR result showed that 5-AZA-CdR significantly increased MAGED4 mRNA (19-fold) in SHG44 instead of A172 (Figure 4A). To assess whether the induction of MAGED4 expression observed at mRNA level was followed by the production of the respective protein, Western blotting were performed. As shown in Figure 4B, 5-AZA-CdR treatment had no effect on MAGED4 protein in both SHG44 and A172. Due to containing many methylated CpG sites in the region 2 of MAGED4 promoter before treatment of 5-AZA-CdR, we choosed this region for further analysis of 5-AZA-CdR influence. Although 5-AZA-CdR treatment resulted in reduction of the overall methylation of this region in both SHG44 and A172, statistic significance was only present in SHG44, not in A172 (Figure 5A). Interestingly, after 5-AZA-CdR treatment on SHG44, some CpG sites (CpG23, CpG27, CpG29 and CpG36) demonstrated complete demethylation with statistic significance (Figure 5B).

Discussion

Studies evaluating the MAGED4 expression in normal tissues and glioma are limited (Sasaki et al., 2001; Kramer et al., 2005). This study demonstrated by use of conventional RT-PCR that normal tissues express MAGED4 mRNA in more than half samples tested. It was unexpected as a previous report (Sasaki et al., 2001), in which a panel of normal tissues was tested and shown

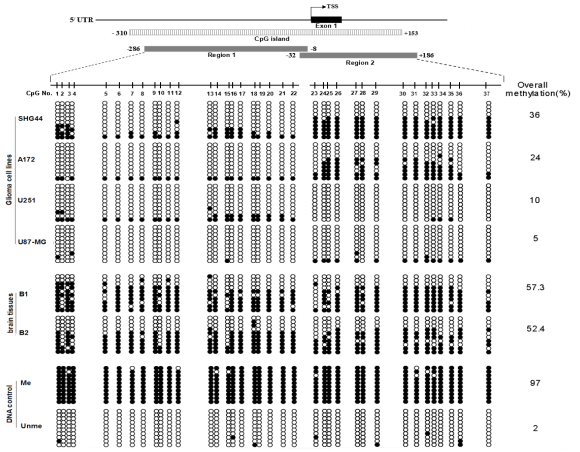

Figure 3. DNA Methylation Analysis of MAGED4 Promoter Region. The right arrow indicates transcriptional start site (TSS). The black box represents the first exon and shaded box delineates the CpG island. Two parts of the CpG island are indicated as grey boxes (region 1 and region 2), in which total of 37 CpG site (vertical line) was analyzed by bisulfite sequencing. Bisulfite converted DNA served as methylation-positive control (Me) and unmethylation-positive control (Unme), respectively. Open circles indicate unmethylated CpG, while filled circles represent methylated CpG

Figure 4A

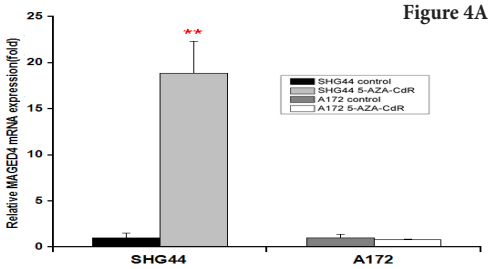

Figure 4B

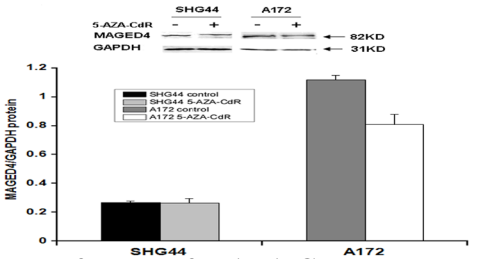

Figure 4. Influence of 5-AZA-CdR Treatment on MAGED4 Expression Levels. Treated cell lines included SHG44 and A172. qRT-PCR (A) and Western blot (B) analysis of MAGED4 expression in cells before and after 5-AZA-CdR treatment. MAGED4 expression was normalized to GAPDH. The mean values from triplicate data points are plotted. Error bars indicate+s.e. $^{**}p<0.01$.

Figure 5A

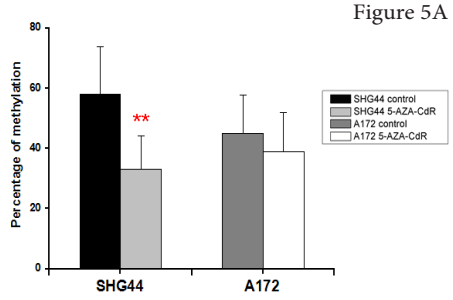

Figure 5B

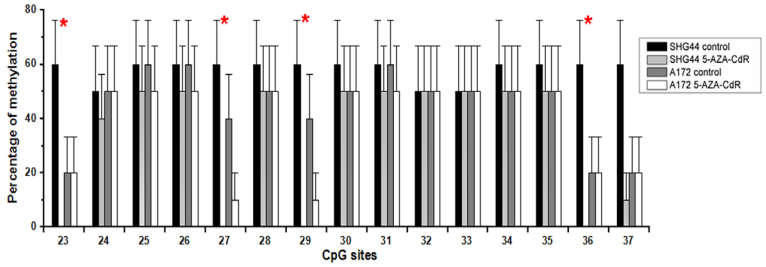

Figure 5. Influence of 5-AZA-CdR Treatment on the Methylation Status of Region 2 in CpG Island of MAGED4 Promoter Region. A) Overall percentages of methylation. **B)** Methylation percentages of individual CpG site. Error bars indicate+s.e. $^{**}p<0.01$; $^{*}p<0.05$.

only brain and ovary expressing MAGED4 mRNA. The discrepancy may caused by different samples tested and method used. In general, RT-PCR analysis applied in present study turned out to be more sensitive than the northern blot used by Sasaki et al. (2001). Therefore, low level MAGED4 mRNA in normal tissues may be easy to detecte by RT-PCR instead of northern blot. Since so many normal tissues expressed MAGED4 mRNA, it is interesting to note that how the expression level of MAGED4 mRNA was. Through qRT-PCR, MAGED4 demonstrated a very low level of expression in normal tissues with MAGED4 mRNA. Kramer et al. (2005) have examined a panel of normal tissues and comfirmed that most normal tissues did not express MAGED4 mRNA, even if some normal tissues expressed MAGED4 mRNA, compared to glioma, they also showed low MAGED4 expression. In our study, many tissue types were different with previous reports (Sasaki et al., 2001; Kramer et al., 2005). Furthermore, all of normal tissues in our test declared negative for MAGED4 protein. Our results add to the limited data on expression of MAGED4 in nornal adult tissues.

It was reported that MAGED4 is overexpressed in many human malignancies, including glioma, kidney cancer, lung cancer, breast cancer and oral squamous carcinoma (Sasaki et al., 2001; Kramer et al., 2005; Ito et al., 2006; Germano et al., 2011; Chong et al., 2012). Although all of glioblastomas tested by previous reports (Sasaki et al., 2001; Kramer et al., 2005) is positive for the MAGED4 mRNA, the number tested is too limited and lack of information for protein expression. For enlarging the spectrum of gliomas where MAGED4 is aberrantly expressed, glioma tissues and cell lines were assessed in the present study. We found that most of glioma tissues and cell lines exhibited higher level of MAGED4 mRNA than normal tissues. Our study also first present the protein expression profile of MAGED4 with a high frequency in both low-grade astrocytoma and high-grade astrocytoma. This suggested that MAGED4 may potentially be used as a promising target for glioma immunotherapy. Nevertheless, heterogeneous intratumor expression of MAGED4 was observed, which may hamper the effectiveness of MAGED4-based glioma immunotherapy due to lack target for cytotoxic lymphocytes. This phenomenon is also frequent in other tumor associated antigen (TAA) including MAGE family (dos Santos et al., 2000; Dhodapkar et al., 2003; Meek et al., 2012). To overcome this obstacle the strategy for up-regulated TAA is need to consider.

Recent studies (Weber et al., 1994; Sigalotti et al., 2004; Wischnewski et al., 2006) have shown that epigenetic manipulation could enhance TAA expression including MAGE family, which is based on these TAA genes commonly with hypermethylated CpG sites in promoter region. Here, we report at the first time the methylation status of MAGED4. Our results demonstrated the CpG sites of MAGED4 promoter region were normally methylated in brain tissues, but was aberrantly demethylated in glioma cell lines. Moreover, the overall methylation rate of MAGED4 promoter region was correlated with mRNA expression in glioma cell lines as well as brain tissues, of which U87-MG expressed the highest level of MAGED4 mRNA, while SHG44 and brain tissues were absent of MAGED4 mRNA. Thus, it is suggested that MAGED4 hypomethylation may be a tumor-associated event and contribute to the induction of MAGED4 expression in gliomas.

To understand the activation of MAGED4 in glioma cells is related to the DNA methylation, glioma cells (SHG44 and A172) were treated with 5-AZA-CdR, a DNA demethylating agent which has been use to upregulated to the expression of genes silenced by the hypermethylation of their promoter (Weber et al., 1994; Sigalotti et al., 2002). Our result showed the reaction to 5-AZA-CdR in the two cells were various. The MAGED4-negative cell (SHG44) was sensitive to the 5-AZA-CdR, resulting in striking increase of MAGED4 mRNA. Whereas MAGED4-positive cell (A172) failed to induce more MAGED4 mRNA after adminstration of the agent. It is similar to the previous report, in which Coral et al. (2002) revealed that 5-AZA-CdR could reactivate other member of MAGE family (MAGE-1, -2, -3 and -4) in these gene-negative renal carcinoma cells, but had no effect on these gene-positive cells. The different respond to the 5-AZA-CdR may be due to the different genetic background of these cells. In addition, Our results also showed that MAGED4 protein expression was not correspondingly upregulated despite MAGED4 mRNA increase after 5-AZA-CdR treatment in SHG44. The explanation might be followings: limited time to treat cells with 5-AZA-CdR, and quick degradation of translated protein. Future study may set different time course to assay the protein induce.

To better explain the fact that increase of MAGED4 mRNA after treatment of 5-AZA-CdR in cells was related to the DNA demethylation, we analyzed MAGED4 promoter region 2 with many methylated CpG sites. The results demonstrated the methylation frequency in this region was apparently decrease in SHG44, which further supports the expression mechanism of MAGED4 is at least mediated by the DNA methylation. It has been reported that not all the CpG sites are crucial for regulating gene expression (Zhang et al., 2004). In the present study, we found CpG23, CpG27, CpG29 and CpG36 site were completely demethylated after 5-AZA-CdR treatment in SHG44. This result will probably lay the foundation for further study of crucial CpG sites controling MAGED4 expression in future.

In conclusion, MAGED4 is highly expressed in glioma, particulaly in high-grade glioma (glioblastoma). DNA methylation in MAGED4 promoter region is an important mechanism in regulation of MAGED4 expression. The efficacy of 5-AZA-CdR treatment on the MAGED4 expression was comfirmed via promoter demethylation in MAGED4-negative glioma cells. It suggests MAGED4 may be used as a potencial target for glioma immunotherapy with 5-AZA-CdR to induce MAGED4 and eliminate the heterogeneous intratumor expression.

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